Journal of Chromatography, 359 (1986) 265–274
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 884

SEPARATION OF DNA RESTRICTION FRAGMENTS BY ION-PAIR CHROMATOGRAPHY

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SUMMARY

The separation of restriction endonuclease fragments of DNA on columns of Pharmacia PepRPC™ (C_2/C_{18}) has been studied. The effect of different concentrations of triethylammonium or tetrabutylammonium salts as ion-pairing reagents, as well as of physical parameters, such as flow-rate and sample load, has been investigated. With the use of triethylammonium buffers, removed by evaporation under vacuum, separated fragments were recovered in yields of 68%. Isolated fragments were accessible to further cleavage with restriction enzymes. Resolution of fragments ranging from 10 to 3000 base pairs depended primarily upon molecular size.

INTRODUCTION

The need for convenient methods for preparing DNA restriction fragments in recombinant DNA work has led to several attempts at complementing preparative gel electrophoresis with high-performance liquid chromatography (HPLC).

Ion-exchange chromatography (IEC) was first explored with the so-called RPC-5 columns[1] and later with commercial columns like TSK 5-PW DEAE[2], Nucleogen-4000 DEAE[3], Mono Q and Mono P[4]. Separations of fragments within a wide range of size have been reported, but resolution of fragments larger than a few hundred base pairs generally declined rapidly. DNA fragments smaller than 300 base pairs have been separated by high-performance gel filtration on Superose[5] or TSK-SW[6] gels. Resolution is lower than obtained by IEC, but it is a convenient method of separation. Fractionations of larger fragments and plasmids on Sephacryl[7] or TSK-PW[8] have been reported. Successful chromatographic separations of DNA fragments as large as 30 000 base pairs have been performed by partition chromatography[9] but this technique is time-consuming and quite laborious.

Ion-pair chromatography is a well-established technique for separating mono- and oligonucleotides[10]. The separation mechanism has been discussed in detail[11]. In an early paper[12], reversed-phase chromatography (RPC) on Kel F powder demonstrated the possibility of separating restriction fragments by the ion-pairing technique, but attempts to transfer the results to commercial RPC materials was not successful.

In the present paper, we have investigated the basic parameters for obtaining efficient separations of double-stranded DNA on PepRPC, a 5-μm, 100-Å C_2/C_{18}

0021-9673/86/$03.50

material, with the ion-pairing technique. Emphasis has been placed on resolving fragments larger than 500 base pairs, in order to expand the separation range compared to ion exchangers, and on the use of volatile buffers to avoid the risk of losing material in precipitation techniques.

EXPERIMENTAL

Chromatography

All chromatographic and electrophoretic experiments were performed with Pharmacia (Uppsala, Sweden) equipment and chemicals according to the manuals supplied. A FPLC® system was equipped with two gradient mixers in series, a V-7 valve for loop injection and a UV-1 monitor with a HR flow-cell for detection at 254 nm. The column was a PepRPC (C_2/C_{18}) HR 5/5 (dimensions 5 cm × 5 mm I.D.), and fractions were collected with a FRAC-100 fraction collector.

Resolution data (R_s) were calculated assuming the peaks to be gaussian. The variance was calculated from estimation of the width at half peak-height.

Electrophoresis

DNA fractions were analysed on gradient PAA 2/16 gels in a buffer of 40 m*M* Tris, 20 m*M* sodium acetate and 2 m*M* EDTA (pH 8.3), with a GE-2/4 vertical gel electrophoresis system. To visualize fragments, the gels were stained with ethidium bromide[13] and inspected under UV light.

Eluents

All buffers were made up from chemicals and solvents of analytical-reagent grade from commercial sources and from high-purity water (Milli Q-system, Millipore, Västra Frölunda, Sweden). To eliminate impurities in buffer salts, stock solutions without acetonitrile were purified by pumping them through a column filled with PepRPC (C_2/C_{18}).

DNA restriction fragments: sample preparation

DNA pBR322 or φX-174 RF (Pharmacia) was cleaved with the required endonuclease restriction enzyme (Hae II, Hae III, Hinf I, Taq I or Alu I) according to the supplier's instructions (Pharmacia) or according to Maniatis *et al.*[13]. After diluting the digest with an equal volume of the starting buffer, the sample was injected without further purification.

DNA concentration: desalting

Fractions containing the desired peak were pooled for electrophoretic analysis, quantitative analysis by fluorescence spectroscopy, or further digestion with endonuclease restriction enzymes. When buffers were made from non-volatile quaternary amine salts, the DNA was precipitated with ethanol according to Maniatis *et al.*[13]. Fractions from chromatography with triethylamine acetic acid buffers were frozen at −70°C and then lyophilized or evaporated directly at room temperature with a Speed VAC concentrator (Savant Instruments, New York, U.S.A.). To avoid losses of DNA due to adsorption, all tubes were silanized[13] before use.

Fluorescence spectroscopy

After evaporation of pooled fractions, the fluorescence measurements were made in 10 m*M* Tris–HCl, 1 m*M* EDTA, and 0.1 *M* sodium chloride (pH 8.0) containing 10 μg/ml propidium iodide with a Shimadzu RF-540 spectrofluorophotometer (Shimadzu, Kyoto, Japan). The emission and absorption wavelengths were 620 and 545 nm, respectively. Samples were measured against known amounts of Hinf I-digested pBR322.

RESULTS AND DISCUSSION

Gradient composition

To investigate the effect of the concentration of different amines on the chromatographic behaviour of restriction fragments, tetrabutylammonium bromide and triethylammonium acetate were chosen as ion-pairing reagents. Tetrabutylammonium salts are commonly used in ion-pair chromatography, but triethylammonium acetate was preferred because it can be removed by evaporation. To stabilize the pH at 6.5, the tetrabutylammonium salt was mixed with 0.03 *M* phosphate buffer. The test mixture was a Hae III digest of φX-174 RF, which contains fragments with 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, and 1353 base pairs (Fig. 1).

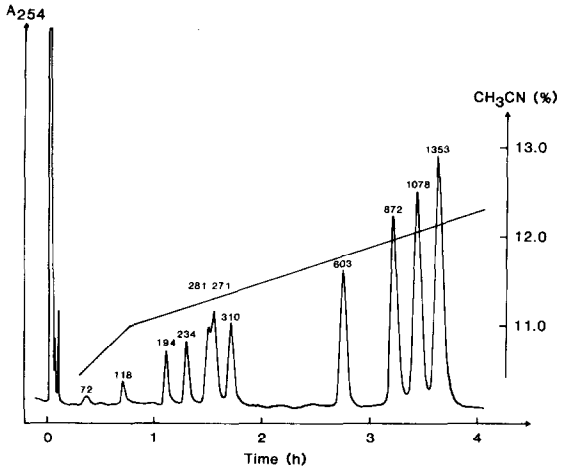

Fig. 1. Separation on PepRPC of DNA restriction fragments from φX-174 RF (10 μg), cleaved with Hae III. Buffer, 50 m*M* triethylammonium acetate (pH 6.5); flow-rate, 0.4 ml/min, gradient, 10.0–11.0% acetonitrile in 50 min; 11.0–12.2% in 180 min. Peaks were assigned according to PAGE.

As shown in Fig. 2, a considerably higher percentage of acetonitrile, together with a lower concentration of the tetrabutylammonium salt, was needed to elute fragments than when triethylammonium acetate was used. Resolution of the fragments with 1078 and 1353 base pairs was almost equivalent and had an optimum at amine concentrations of about 12 and 40 m*M*, respectively, as shown in Fig. 3. At higher concentrations, both resolution and peak shape deteriorated, especially for smaller fragments. With concentrations of triethylammonium acetate below 20 m*M*,

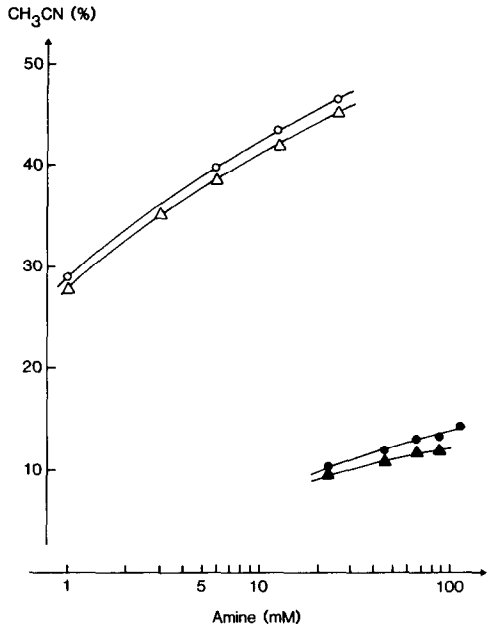

Fig. 2. Percentage of acetonitrile needed for elution of restriction fragments from φX-174 RF (10 μg) cleaved with Hae III, as a function of amine concentration. (○,△) 1353 and 234 base pairs, respectively, with tetrabutylammonium bromide in 0.03 *M* phosphate buffer (pH 6.5); flow-rate, 0.4 ml/min; gradient slope, 0.017% acetonitrile/ml. (●,▲) 1353 and 234 base pairs, respectively, in triethylammonium acetate (pH 6.5); flow-rate, 0.4 ml/min; gradient slope, 0.017% acetonitrile/ml. For each individual chromatogram, the gradient range was 3%.

reproducibility was poor, probably because of ionic interaction phenomena related to the weak buffer capacity.

Flow-rate and gradient slope

The resolution of larger fragments was greatly influenced by the flow-rate (Fig. 4). In our experience, for separations of fragments larger than 1500 base pairs, flow-rates below 0.25 ml/min should be used and for molecules of 500–1500 base pairs, flow-rates less than 0.5 ml/min should be used. With fragments smaller than 500 base pairs, flow-rates greater than 0.5 ml/min can be used without serious impairment of resolution. The gradients used were generally very shallow, in the range of 0.01 to 0.1 percentage unit increase of acetonitrile per ml of eluent (0.01–0.1%/ml). Chromatograms of a pBR322/Hinf I digest with gradient slopes of 0.029 and 0.013%/ml are shown in Figs. 5 and 6, respectively. To obtain reproducible results when the overall range of concentration in the gradient is small, gradients should be prepared from solutions which represent the limits of the gradient. For example, a gradient from 10 to 15% acetonitrile should be formed from solutions of 10% acetonitrile and 15% acetonitrile, rather than directly from 0% acetonitrile and 100% actonitrile.

Load capacity

PepRPC is based on a silica matrix with a pore distribution around 100 Å.

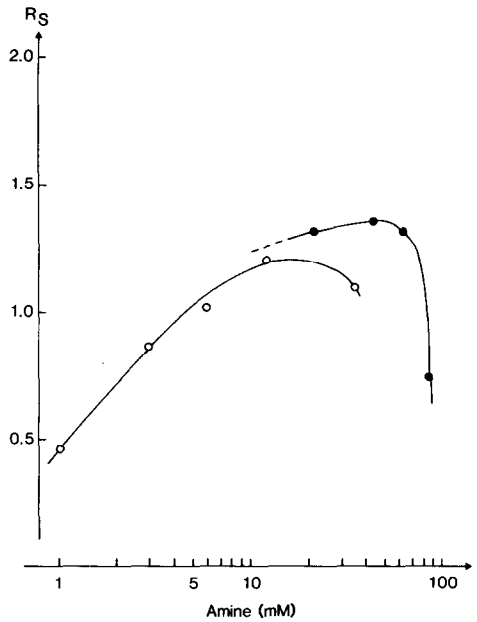

Fig. 3. Resolution of fragments with 1078 and 1353 base pairs from φX-174 RF (10 μg) cleaved with Hae III, as a function of the concentration of tetrabutylammonium bromide (○) in 0.03 *M* phosphate buffer (pH 6.5) or triethylammonium acetate (●) (pH 6.5); flow-rate, 0.4 ml/min; gradient slope, 0.017% acetonitrile/ml.

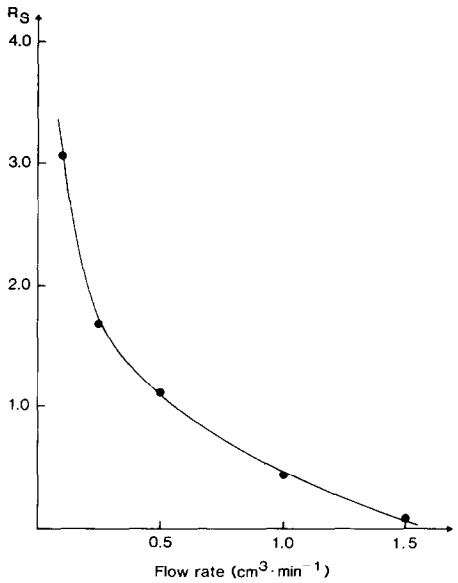

Fig. 4. Resolution of fragments with 1078 and 1353 base pairs from φX-174 RF (10 μg) cleaved with Hae III, as a function of flow-rate. Buffer, 100 m*M* triethylammonium acetate (pH 6.5); gradient, 12–15% acetonitrile; gradient slope, 0.017%/ml.

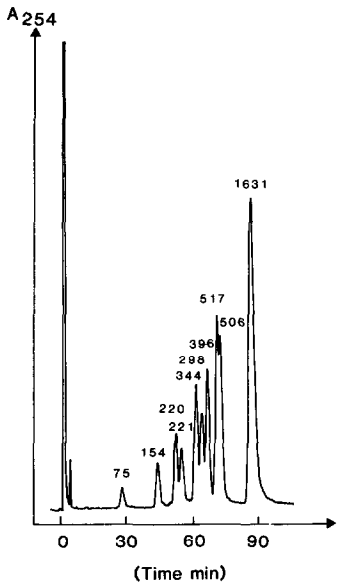

Fig. 5. Separation on PepRPC of DNA restriction fragments from pBR322 (5 μg) cleaved with Hinf I. Buffer, 30 m*M* triethylammonium acetate (pH 6.5); flow-rate, 0.8 ml/min, gradient, 9.5–12.0% acetonitrile in 110 min. Peaks were assigned according to PAGE.

Thus, it is expected that all fragments larger than approximately 100 base pairs should be totally excluded from the inner volume. This fact should decrease the load capacity below that of materials with larger pore diameter. However, only small changes in performance were observed when 10-μg and 100-μg samples of the pBR322/Hinf I digest were compared under identical conditions, as shown in Fig. 6.

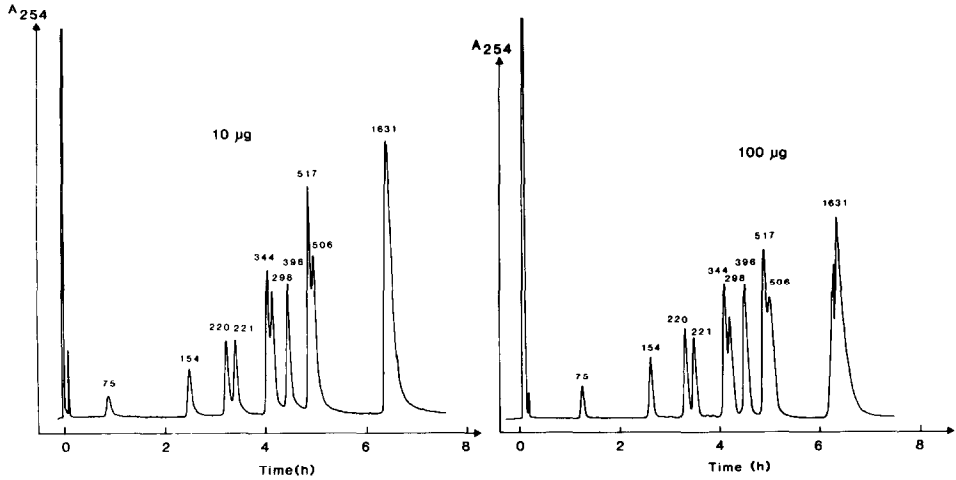

Fig. 6. Separation on PepRPC of DNA restriction fragments from pBR322 (10 μg and 100 μg) cleaved with Hinf I. Buffer, 30 m*M* triethylammonium acetate (pH 6.5); flow-rate, 0.4 ml/min; gradient, 9.5–12% acetonitrile in 480 min.

DNA recovery and quality

Recoveries of different fragments were determined by injecting a 25-μg sample of Hinf I/pBR322 digest. After collection and evaporation of the pooled fractions, the isolated material was treated with a solution of propidium iodide, and the amount of DNA was determined by fluorescence spectroscopy[14]. The total recovery was 68%; the recovery of each fragment is shown in Table I. Integration of the chromatogram (Fig. 6) indicated that losses of the individual fragments were due to adsorption of material after chromatography.

TABLE I

RECOVERY OF DNA FRAGMENTS

Separation on PepRPC of DNA restriction fragments from pBR322 (25 μg), cleaved with Hinf I. Buffer, 30 m*M* triethylammonium acetate (pH 6.5); flow-rate, 0.4 ml/min; gradient, 9.5–12% acetontrile in 480 min.

Fragment (base pairs)	*Recovery (%)*
75	35
154	43
220 + 221	75
298 + 344	75
396	86
506 + 517	72
1631	60

Isolated DNA fractions were routinely analysed and identified with polyacrylamide gel electrophoresis (PAGE). Very little cross-contamination was observed. To ensure that no enzyme inhibitor had accumulated, evaporated fractions were tested for further cleavage of fragments by restriction enzymes. Thus, the 1631 base-pair fragment was digested with Taq I restriction enzyme, and the cleavage was confirmed by reinjecting the digest on the PepRPC column under the conditions given in Fig. 5.

In a separate experiment, the isolated fragments tentatively assumed to be 220 and 221 base pairs and which did not separate on PAGE, were treated with the restriction enzyme Alu I. This enzyme is expected to split only the 220 base-pair fragment. Each digest was rechromatographed, as shown in Fig. 7, and only one fragment was cleaved further, confirming this to be the 220 base pair fragment.

Separation mechanism

With minor exeptions (*cf.* Figs. 1 and 5), resolution was generally a function of size (Fig. 8). This is in contrast to earlier work[2–4] with ion exchangers, where fragments with a high proportion of the bases adenine and thymine are retarded more than expected. As illustrated in Fig. 9, with a pBR322/Hae III digest the order of elution is not as sensitive to the base composition. From ion exchangers[2,4], the 458 base pair fragment is eluted later than the peaks corresponding to 504 and 540 base pairs.

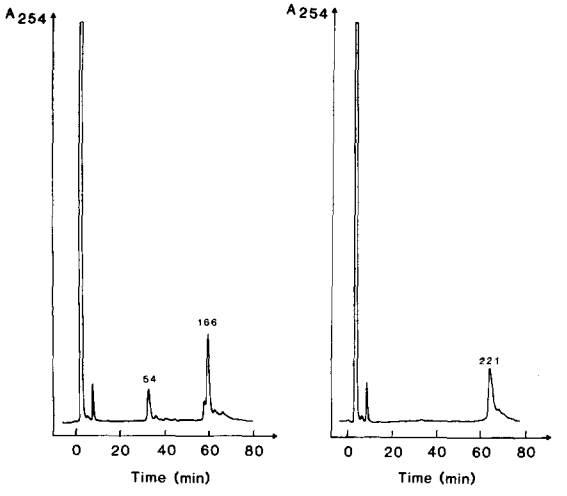

Fig. 7. Separation on PepRPC of isolated 220 and 221 base-pair fragments digested with Alu I. Buffer, 30 m*M* triethylammonium acetate (pH 6.5); flow-rate, 0.5 ml/min; gradient 9.0–12.0% acetonitrile in 60 min.

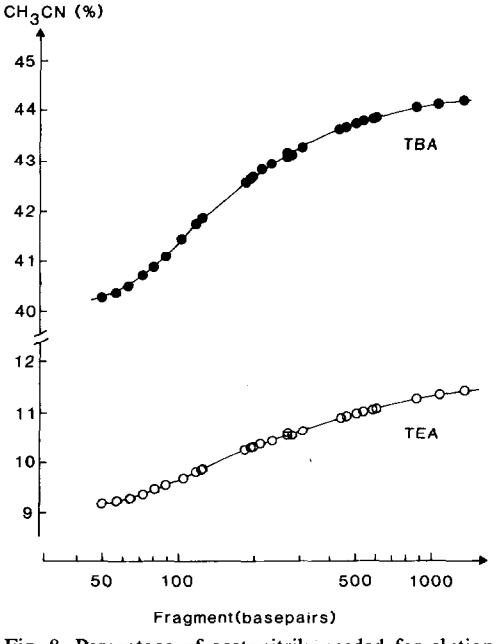

Fig. 8. Percentage of acetonitrile needed for elution of restriction fragments from Hae III digests of pBR322 (10 μg) and φX-174 RF (10 μg). (●) With 12 m*M* tetrabutylammonium bromide in 0.03 *M* phosphate buffer (pH 6.5); flow-rate, 0.3 ml/min; gradient, 40–45% acetonitrile in 16 h. (○) With 30 m*M* triethylammonium acetate (pH 6.5); flow-rate, 0.3 ml/min; gradient, 9–12% acetonitrile in 13 h.

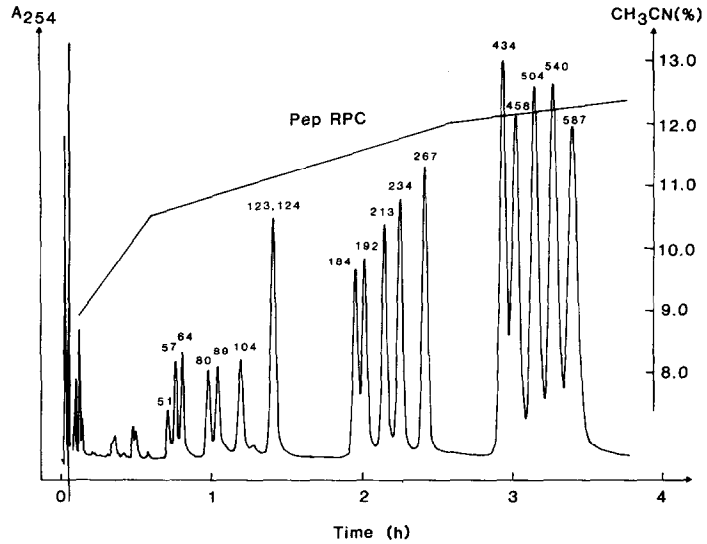

Fig. 9. Separation on PepRPC of DNA restriction fragments from pBR322 (10 μg) cleaved with Hae III. Buffer, 40 m*M* triethylammonium acetate (pH 6.5); flow-rate, 0.5 ml/min; gradient, 8.5–10.5% acetonitrile in 40 min; 10.5–12.0% in 120 min; then 12.0–12.3% in 70 min. Peaks were assigned according to PAGE. Fragments with 7, 11, 18 and 21 base pairs were not identified.

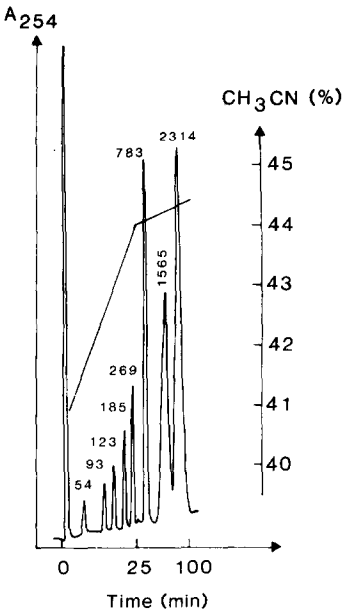

Fig. 10. Separation on PepRPC of DNA restriction fragments from φX-174 RF (5 μg) cleaved with Hae II. Buffer, 12 m*M* tetrabutylammonium bromide in 0.03 *M* phosphate buffer (pH 6.5); gradient and flow-rate, 40.5–44.0% acetonitrile in 25 min, 1.0 ml/min; 44.0–44.3% acetonitrile in 75 min, 0.2 ml/min.

Larger fragments

Fragments up to a size of 2000 base pairs can be separated within 2–4 h. An example is given in Fig. 10. It is also possible to separate even larger fragments. However, with these larger fragments, peak deterioration is often observed even at low flow-rates. To obtain satisfactory results, longer separation times must be used.

Our results indicate that ion-pair chromatography is an efficient and convenient method for the preparative separation of double-stranded DNA. The resolution of fragments up to 500 base pairs in length is comparable with that obtained by ion-exchange chromatography, and the resolution of larger fragments is clearly superior. Although gel electrophoresis often gives better resolution, ion-pair chromatography with the use of volatile buffers may be preferable, being a convenient way of isolating specific fragments for further use in recombinant DNA techniques.

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